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PRINCIPAL INVESTIGATOR: Dr. John Shoffner

CONTRACTING ORGANIZATION: Georgia State University Research Foundation
Atlanta, GA 30303

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Dr. John Shoffner

E-Mail: jshoffner@gsu.edu

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14. ABSTRACT

The purpose of this study is to define correlations between indices of brain dysfunction, such as functional MRI (fMRI) and neuropsychological testing abnormalities, with oxidative phosphorylation (OXPHOS) defects in children with autistic spectrum disorders (ASD). This study is an essential step in identifying such a phenotypic subtype, being able to perform large-scale epidemiological studies using more widely available measures, and ultimately being able to implement clinical trials for new pharmaceutical agents emerging for treatment of the OXPHOS defects which could significantly improve the functioning of children with ASD with this defect. In the current year, we have continued enrolling subjects into the study and have also begun gathering data on these subjects. We have begun analyzing these data, however, data acquisition is ongoing and larger numbers of subjects are needed before we will be able to draw meaningful conclusions from this study.

15. SUBJECT TERMS
Autism, functional MRI, mitochondria, mitochondrial disease

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Introduction

Although the precise frequency of mitochondrial defects in autism are not known, it is hypothesized that significant numbers of individuals with autism and autistic spectrum disorders (ASD) harbor oxidative phosphorylation (OXPHOS)defects important to ASD disease pathogenesis and/or functioning. These OXPHOS defects are identifiable in muscle, fibroblasts and EBV transformed lymphocytes. Pathogenic mutations in OXPHOS genes are predicted to be observed in patients with ASD at higher rates than in the general population. We propose that these OXPHOS defects correlate with indices of brain dysfunction such as functional MRI (fMRI) and neuropsychological testing abnormalities and define a specific subtype of children with ASD. This study is an essential step in identifying such a phenotypic subtype, being able to perform large-scale epidemiological studies using more widely available measures, and ultimately being able to implement clinical trials for new pharmaceutical agents emerging for treatment of the OXPHOS defects which could significantly improve the functioning of children with ASD with this defect.

Body

Brief background:

Mitochondria are cytoplasmic structures with an inner and outer membrane separated by an intermembrane space. Oxidative phosphorylation (OXPHOS) is critical to cellular function as the primary source for energy (ATP) in most cell types, the control point for cellular redox, and as a control point for essential metabolic and signaling pathways that range from the synthesis of pyrimidines for the regulation of apoptosis. Substrates for ATP generation are derived primarily from glycolysis and fatty acid oxidation.

OXPHOS uses about 95% of the oxygen delivered to tissues, producing most of the ATP required by cells. Expression of genes involved in the OXPHOS pathway and the assembly of the five OXPHOS enzyme complexes Complex I (CI), Complex II (CII), Complex III (CIII, CIV and CV) within the inner mitochondrial membrane is a highly ordered and coordinated process directed by 37 genes in the mitochondrial DNA (mtDNA) and as many as 1,500 genes in the nuclear DNA (nDNA)[1,2].

Over 50 pediatric and adult diseases are caused by mutations in a heterogeneous array of OXPHOS genes coded by either the nDNA or the mtDNA. Genetic defects producing mitochondrial dysfunction include: (1) inherited mutations in

nDNA or mtDNA genes. (2) Sporadic mutations occurring during embryogenesis that are systemic or confined to specific tissues such as skeletal muscle. (3) Somatic mutations occurring through life due to aging, free radical damage, and exposure to environmental toxins or certain medications. Defects in OXPHOS have a broad array of cellular consequences including abnormal cellular calcium (Ca^{2+}) regulation, impaired ATP generation, enhanced apoptosis, and increased free radical production [3-6]. In fibroblast cell lines harboring pathogenic mutations in CI genes, CI dysfunction causes depolarization of the mitochondrial membrane potential, resulting in a decreased supply of mitochondrial ATP to the Ca^{2+}-ATPases that control intracellular Ca^{2+} stores. Ca^{2+} content of these stores is then reduced, particularly in the endoplasmic reticulum [7]. Defects in any of these functions can lead to disease.

Most energy used for neuronal activity is expended as a result of the postsynaptic neuronal depolarization and to a lesser extent the action potentials generated [8]. OXPHOS uses approximately 95% of the oxygen delivered to tissues, thus making fMRI an important tool for non-invasive investigation of mitochondrial dysfunction. The energy cost arises from information transfer and its integration postsynaptically. Substrate delivery for energy metabolism is increased along with increased local blood flow in conjunction with to neurotransmitter action (local signaling). Reduced oxygen extraction as occurs with mitochondrial dysfunction leads to an increase in the ratio of oxy- to deoxyhemoglobin during neuronal activation.

Mitochondrial disease produces detectable fMRI abnormalities. For example, in Friedreich ataxia, a mitochondrial disease caused by abnormal iron incorporation into OXPHOS enzyme active centers [9], fMRI studies during motor tasks show cortical hypoactivity in a pattern consistent with the mitochondrial dysfunction [10]. fMRI abnormalities are well described in ASD and correlate with clinical features. Cortical hypoactivity in ASD includes fusiform gyrus which is associated with poor facial recognition [11] and anterior cingulate cortex which is related to inflexible and repetitive behavior [12]. Although the neuroanatomical substrates of the ASD phenotype are characterized, studies correlating fMRI findings with biochemical or molecular defects are lacking.

Task 1: (Specific Aim I, human subjects, Months 1-30) Assessment of Neuropsychological Functioning. In order to make appropriate statistical analyses among neuropsychological data, fMRI data and laboratory data, the data analysis will extend to the end of the study (month 36).

In order to complete this task, detailed clinical characterization and correlation with biochemical data on each patient must be performed. In the current year, we have continued to clinically evaluate subjects that were identified during the first year of this study. A detailed description of the data that we used for stratification of these subjects was presented in the first annual review and will not be thoroughly discussed here. Briefly, a number of biochemical tests on muscle samples from these subjects had been performed and these data were utilized to classify the subjects as having or NOT having mitochondrial dysfunction. A subset of these data is summarized in Table 1.

Table 1: Distribution of OXPHOS ABNORMALITIES in ASD patients. The data listed are used for stratification into the two groups discussed above.

Biochemical OXPHOS Test	Results Summary (% abnormal)
OXPHOS Enzymology (Complexes I-IV)	63.6% (42/66)
High resolution respirometry (live cells)	28.6% (10/35)
Muscle CoQ10 level	2.7% (1/36)
OXPHOS subunit Western blot	34.1% (14/41)
Blue and Clear Native gel testing	1.Oxphos Supercomplex formation: 50% (19/38) 2. Monomeric Enzyme Assembly: 52.6% (20/38) 3. Clear Native Gel of Intact OXPHOS enzymes: Abnormal enzyme activity in 36.8% (14/38)

During the past year (year 2) we have received fourteen new consents, and have continued work on the original ten subjects who consented during Year 1. For Year 2 consents, three came from Georgia subjects and eleven came from out of state subjects.

Over the year, we have completed 18 of the 24 Autism Diagnostic Interview – Revised (ADI-R) for the total sample (no ADI-Rs were completed during Year 1), with two more currently scheduled. Of these 18 ADI-R interviews completed, five did not currently meet ADI-R study criteria on this parent interview,

although all had strong documented histories of professional ASD diagnoses when they were younger.

In addition, we completed the in-person diagnostic play session (ADOS) on nine subjects. Interestingly, of the four subjects whose parent ratings did not meet criteria for ASD, two were classified as ASD using the ADOS play session observational measure. Furthermore, of the thirteen children whose parent's ratings did meet the ADI-R criteria, five completed the ADOS diagnostic play session and two met its criteria, but three did not. This pattern of different results from different measures on children with histories of the ASD diagnosis is presented in Table 2. This complex pattern of documented history, parent report, and diagnostic play session results, has resulted in only two children with mitochondrial disease meeting both the ADI-R and ADOS criteria as proposed in our original study protocol, a very limited and unexpected yield from the eighteen subjects who have had the ADI-R, and the nine who have also had the ADOS evaluation. Complete summaries are presented in Figure 1 and Tables 2-4.

Table 2: Summary of ADI-R and ADOS progress in the 24 subjects that are enrolled in the study.

	consent obtained	ADI in progress	ADI completed	ADOS completed	meets full study criteria
Year 1 as of 6/30/2011	10	2	0	0	0
Year 2 as of 6/30/2012	14	1	13	5	2
Totals	24	1	18	9	2
					doesn't meet study criteria
					7

Table 3: ADI-R and ADOS results summary for the subjects that have participated to date.

Subject Characteristics (avg (std, range)	Total Sample ADI-R results (n=13)
Age (in years)	8.8 (6-11)
% Females	
ADI-R Social	15.2 (9.4, 1-27)
ADI-R Communication	13.2 (6.6, 4-24)
ADI-R Repetitive Stereotyped Behavior	4.1 (3.5, 0-9)
ADI-R Abnormal or Developmental Problems Evident at or before 36 months	2.9 (1.8, 0-5)

Figure 1: Flow chart summarizing testing for the 24 subjects that are currently enrolled in the study.

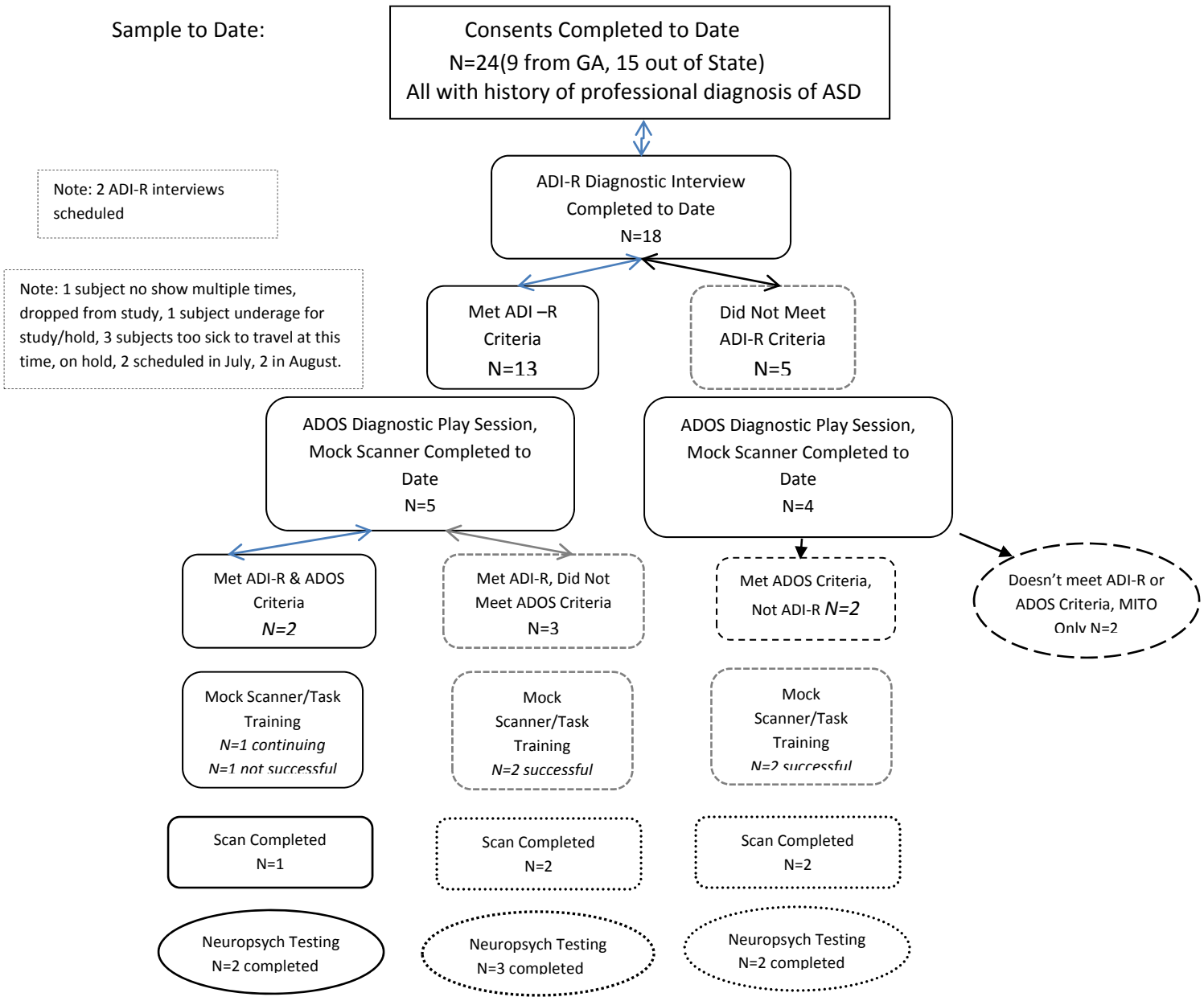


Table 4: fMRI training and participation summary for the study subjects that have participated to date.

	parent measures completed	mock training in progress	mock training completed	Nback training in progress	Nback training completed	NP testing in progress	NP testing completed	scan scheduled	scan completed
Year 1 as of 6/30/2011	0	0	0	0	0	0	0	0	0
Year 2 as of 6/30/2012	5	0	2	0	2	0	2	0	1
Totals	5	2	2	2	2	2	2	1	1

One of the continuing results from this process is that there are a number of cases that continue to have a documented history of receiving autism or ASD diagnoses at younger ages but may have "grown out" of meeting the criteria for current autism/ASD diagnosis. The parents of several of these children report that they are much more "autistic" when they have their periods of sickness. We also know that parents may have various agendas related to rating their children's functioning. Some clearly state their children have been diagnosed with autism in the past but they don't believe that was accurate, while others suggest that they are more impaired at times than when we are seeing them, and their ratings reflect this. In our interactions with these subjects, they all clearly have features which are not completely typical in their interactions and obsessiveness, and many appear classically ASD in presentation clinically, so it is not surprising that they may have been diagnosed with autism by some professional in the past. In addition to studying children with ASD as outlined in the study, it will be important to investigate those that have mitochondrial disease and a diagnosis of ASD at the time their muscle biopsy was performed. As we are reviewing these children for inclusion in this study, we are finding that some have had clear testing and criteria for classification of ASD when their muscle biopsy was performed. However, as they have aged the ASD symptoms abate and they no longer meet criteria for a diagnosis of ASD. This is certainly an interesting subset of children in our group of ASD patients.

Task 2: (Specific Aim II, human subjects, Months 1-30) Neuroimaging: In order to make appropriate statistical analyses among neuropsychological data, fMRI data and laboratory data, the data analysis will extend to the end of the study (month 36).

Data acquisition and analysis will continue throughout the study. To date, we have had two children who met both ADI-R and ADOS diagnostic criteria. One subject completed all aspects of the study, including the fMRI scan during the year. The other completed all aspects except the scan, but we will continue to work with them in the mock scanner to see if they will be able to be successfully scanned in the future.

Additionally, we have had five subjects who met only one of the ADI-R or ADOS criteria. As mentioned above, there are a number of cases that continue to have a documented history of ASD at younger ages but with age no longer meet the diagnostic criteria for ASD. We are currently reviewing the medical records of these subjects in order to see if we can include them in our study. Thus far, we have been able to find clear ASD diagnoses for two of these subjects. Although not listed in the table above, all of these subjects have participated in the fMRI training and are ready to be scanned, if the protocol modification is approved by the IRB (Georgia State and DOD).

Task 3: (Specific Aim III, Banked samples that include muscle, fibroblasts, and EBV transformed lymphocytes) Months 1-30. Although data analysis will be proceeding during the study, the last 6 months are reserved for assessing the data obtained from all specific aims (tasks).

As in our last annual report, we continue to make significant progress on this specific task. These types of analyses are complex, requiring comparison of patients with autism to various categories of normal control cell lines and disease controls.

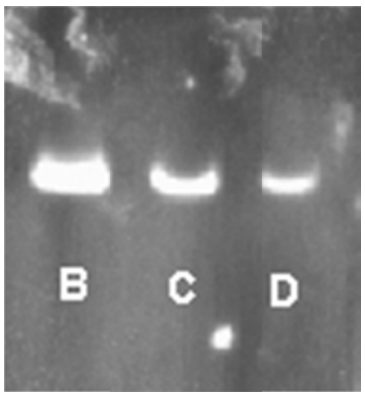
During this reporting period, we have successfully established the reference intervals for fibroblasts and EBV transformed lymphoblasts and have validated these techniques by comparison with patient cell lines that harbor known mitochondrial DNA mutations or nuclear OXPHOS gene mutations (Table 5 and Figure 2). A variety of classes of mutations are being studied in order to understand how the various mitochondrial disease mechanisms affect the results of each test. Sample testing is currently underway and data analysis will be performed when sufficient numbers are obtained. We

expect a majority of this analysis to occur during the last six months of this study.

Table 5: High resolution respirometry normal reference intervals for EBV transformed lymphocytes.

	5%	25%	Mean	75%	95%
Uncoupling Ratio	1.319	1.796	2.046	2.296	2.773
Net Routine Flux Control Ratio	0.316	0.350	0.373	0.397	0.430
Respiratory Control Ratio	7.10	9.28	10.79	12.30	14.47
Leak Flux Control Ratio	0.064	0.083	0.097	0.110	0.129
Phosphorylation Respiratory Control Ratio	0.218	0.253	0.277	0.301	0.335

Figure 2: Clear Native oxidative phosphorylation enzyme activity analysis in fibroblasts. One control sample (B) and two abnormal samples (C and D) are shown. Complex V ATPase activity appears reduced in the two abnormal samples. ABN = abnormal, NL = control.



Task 4: (Specific Aim 4, Banked DNA Analysis) Months 12-30

This task relies heavily on Task 3 in order to determine which genes to analyze for each patient. While sample testing is underway, data analysis will not be performed until sufficient numbers are obtained in order to draw meaningful conclusions. We expect a majority of this analysis to occur during the last six months of this study.

Key Research Accomplishments:

1) Fourteen new subjects have been enrolled in the study during the past year.
2) Eighteen subjects have been evaluated by ADI-R during the past year.
3) Nine subjects have been evaluated by ADOS during the past year.
4) Control ranges have been established for both fibroblasts and EBV transformed lymphocytes in high resolution respirometry, OXPHOS Western blots, and Native gel analysis.
5) Several manuscripts are currently being written that will reference this grant. One paper will be using the early history of these children and their current ADI-R and ADOS results to try to put together a better understanding of how these children are similar/different from ASD children that do not have OXPHOS disease. A second paper will be written on the subjects that do not qualify for this study. It will focus on laboratory and fMRI data in subjects that have OXPHOS disease and do NOT show signs of ASD. This study will provide an interesting and meaningful contrast to the data collected in this study. A third paper is examining relationships across medical indices, biochemical measures, and neurobehavioral functioning in children with mitochondrial disease based on the prediction that impaired functioning of proteins, protein complexes, and cellular respiration, that are all critical in ATP production, will impact neurodevelopment and related neuropsychological processes in these children.

Reportable Outcomes:

None at this juncture in the study.

Conclusions:

We have made significant progress in several aspects of this study during the past year. We have received fourteen new consents and have had our first subject complete all aspects of the study. In addition, we have amended the protocol to expand the age range of subjects. This will allow us to enroll several subjects that were interested in the study, but were unable to participate due to their age. These older subjects are more likely to be able to complete all aspects of the study. Furthermore, we have established reference ranges for several laboratory tests that will allow us to better assess these subjects.

A continuing challenge for the study is the availability of subjects who have to travel a great distance and the extra expenses they incur if they are going to participate in the study. We are developing a process to provide a couple of days of per diem for such subjects by reallocating parts of our budget to meet this need. Hopefully this will be available during the next month, so that parents can plan on visiting in the coming months. We also are seeing a large number of subjects during the summer months when the out of state families can travel more easily.

In summary, we are starting to find some interesting results that may begin to help us understand the similarities/differences between autism/ASD and mitochondrial diseases which appear to be related to developmental changes. The study protocol is a very challenging protocol for these children and their families, but we are progressing and gathering unique data along the way. We are addressing the significant challenges that have arisen in the study. The summer months when these children are out of school is becoming a key time period to meet family scheduling needs because of the time commitment required to be involved and we anticipate making large strides in this study during this time period.

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Appendices:

None.